

Extraction of Glycoprotein Molecule (N-acetylglucosamine) from Chitin by Chitinase Producing Marine Bacteria

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ABSTRACT:

A rapid expansion of livestock's is demanding an adequate supply of efficient, nutritious and inexpensive feed. Biological degradation of crustacean wastes (Chitin) will comfist to feed livestock's. Bacteria play an important role in degrading chitin to N-acetylglucosamine and chitosan, which can then be metabolized to generate energy CO₂, H₂O and NH₃. Isolation and characterization of chitinase producing bacteria have the prime importance to degrade chitin and recycled as various by-products. We isolated two bacterial strains CPB1 and CPB2 from shrimp pond and identified as *Vibrio alginolyticus* and *Pseudomonas aeruginosa* respectively. CPB1 showed better chitinase activity (20µM/ml) than CPB2 after 6 hours of incubation at 37° C. The maximum activity of chitinase for CPB1 was observed at pH 8.0 and CPB2 showed moderate activity at pH 7.0 to 9.0. The extracellular chitinase from two strains were purified from Sephadex G-25 column and run on SDS-PAGE which showed the molecular weights as 60KDa and 86KDa respectively. By fermentation with these bacterial strains or its enzymes, will solubilise chitin to produce glycoprotein molecule and used as livestock's as feed.

Keywords: Chitinase, N-acetylglucosamine, chitin, degradation, protein, livestock

INTRODUCTION

Microbial enzymes have been used in various industries for many centuries. Recently the advent of biotechnology, there has been a growing interest in and demand for enzymes with novel properties. Currently, microbes from terrestrial sources are employed for industrial production of enzymes, although the potential for synthesis of several novel enzymes like phosphatase, arylsulfatase, chitinase, l-asparaginase, l-glutaminase, amylase, protease, lipase, cellulase, urease, and lactamase produced by marine bacteria, cyanobacteria, fungi [1] and actinomycetes [2]. Global annual recovery of chitin from the processing of marine crustacean waste is estimated to be around 37,300 metric tons [3].

Chitin is a widely distributed, Chitin, a homopolymer of N-acetyl-D-glucosamine (Glc- NAc) residues linked by b-1-4 bonds, is the most widespread renewable natural resource following cellulose [4], naturally abundant amino polysaccharide, insoluble in water, alkali, and organic solvents, and slightly soluble in strong acids. Shrimp and crab processing wastes containing chitin, protein, and calcium carbonate are generally pre-treated by the processes of size reduction, deproteination, and demineralization to obtain a chitin suitable for bioconversion or other uses [5,6,7].

Chitinase-producing marine bacteria play an important role in the degradation of chitin in the oceans [8,9] and their properties were studied. Chitinases have been isolated from numerous bacterial sources including *Pseudomonas* sp, *Vibrio furnissii* [10], *Alteromonas* sp. [11], *Vibrio anguillarum*, *Vibrio parahaemolyticus*

[12], *Salinivibrio costicola* [13] and *Microbulbifer degradans* [14]. Moreover *Serratia marcescens* produce numerous chitnase enzymes and isoenzymes that have been characterized at the gene level [15]. Applications of chitinase will results in degradation of chitin molecule from most of the crustacean waste to N-acetyloglucosamine and its hydrolysate can be used as a source for the production of single-cell proteins [16] feed for livestock's.

With this view, the work was scrutinized to isolate the chitinase producing bacteria from the marine ecosystem and characterization of chitinase enzyme. This enzyme may have application in recycling of waste into valuable bioproducts.

MATERIALS AND METHODS

Sample collection and screening of Chitin-degrading Bacteria

Seawater and sediment were collected from the shrimp ponds. Samples were diluted 10 to 1,000 fold in sterile seawater and spread on chitin-containing minimal agar plates (colloidal chitin 12 g, (NH₄)₂SO₄ 2 g, KH₂PO₄ 0.7 g, Na₂HPO₄·7H₂O 0.2 g, FeSO₄·7H₂O 1 mg, MnSO₄·5H₂O 1 mg, agar 15 g, distilled water 500 ml, aged sea water 500 ml, pH 7.0). After incubation for 3-5 days at room temperature, clear-hole forming bacteria were selected as the chitinase producer.

Assay of chitinase activity

Chitinase activity for two bacterial strains (CPB1 and CPB2) was estimated at 1 hour interval of time of incubation. Chitinase activity (U) was determined on the basis of the number of µmoles of released N-acetyloglucosamine is equal to 1 µg of protein. Protein

content was determined by Lowry *et al.*, (1951) method [17].

Table1: Biochemical tests for identification of CPB1 and CPB2 strains

S.No	Biochemical Tests	CPB1	CPB2
1	Indole	+	-
2	Methyl Red	-	-
3	Voges Proskauer	+	-
4	Citrate Utilization	+	+
5	Triple Sugar Iron Gas H ₂ S Production	K/A - -	K/K - -
6	Urease	-	+
7	Catalase	+	-
8	Cytochrome Oxidase	+	+
9	Phenylalanine deaminase	-	-
10	Lysine decarboxylase	-	-
11	Arginine	-	+
12	Ornithine decarboxylase	+	+
13	Nitrate reduction	+	+
14	Esculin	-	-
15	Glucose	+	+
16	Mannitol	-	+
17	Sucrose	-	-
18	Xylose	-	-
19	Trehalose	+	-
20	Lactose	-	-
21	Mannose	+	+

Note: + = Positive, - = Negative, K- Alkaline Slant, A- Acid Butt

Estimation of protein (N-acetylglucosamine)

The amount of protein present in the culture filtrate was qualified through Lowry *et al.*, (1951) method [18]. Mixed 1ml of sample with 5ml of freshly prepared alkaline copper sulphate and incubated at room temperature for 10 mins. To this 0.5ml of Folin Ciocalteu's reagent was added and incubated at room temperature for 20 mins and the absorbance was measured at 660nm. The blank was prepared using the same procedure without sample. The protein content was estimated by calibration with the standard graph.

Effect of pH on the chitinase activity

The effect of pH on the active chitinase producing strain in chitin minimal media after 5 hours of incubation was determined with different pH values by adjusting pH (5 to 10.0) by using standard conditions. The buffer systems used as follows: 0.1 M citrate-phosphate buffer for pH 5.0 and 6.0, 0.1 M phosphate buffer for pH 7.0 and 8.0 and 0.1 M glycine-NaOH buffer for pH 9.0 and 10.0.

Purification of Chitinase

The method was followed by Ohtakara *et al.*, (1979) [19]. The isolates CPB1 and CPB2 were pre-cultured in the same medium as described above without agar for 3 days at 30°C and cell free culture broth (900ml) were precipitated with ammonium sulphate (30%, w/v).

The precipitate was obtained by centrifugation (16,000 \times g, 30 min, 4° C) and suspended in 3 ml of 0.1 M citrate-phosphate buffer (pH 6.0). The suspension was eluted through a Sephadex G-25 column (1 by 6 cm) to remove low molecular compounds and then lyophilized.

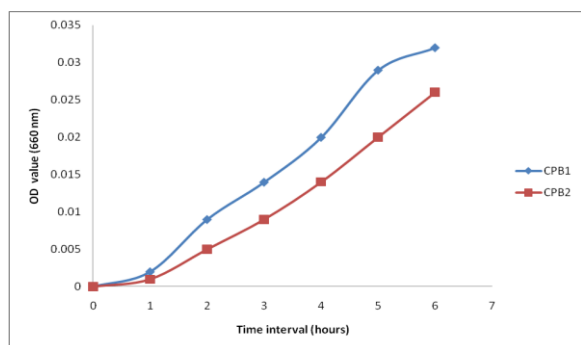
Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The molecular mass of the purified chitinase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel. The sample, obtained from Sephadex G-200 column chromatography and then concentrated by freeze-drying, was applied onto a gel and run at 20 mA. The gel was stained with 0.2% silver nitrate.

RESULTS

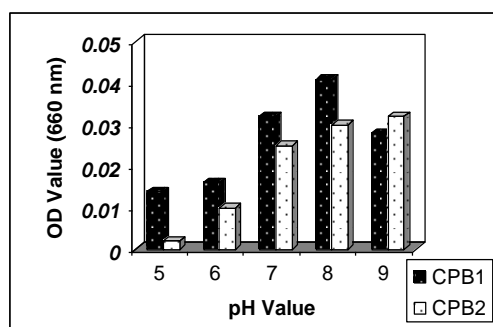
Two different bacterial strains were isolated from shrimp pond sediment with chitinase producing activity through selective agar medium as CPB1 and CPB2 and identified through twenty biochemical characteristics which include indole, methyl red Voges Proskauer test, citrate utilization and some carbohydrate utilization test. This biochemical tests results may distinguish the isolates belongs to be specific group (Table 1.) and identified as *Vibrio alginolyticus* and *Pseudomonas aeruginosa* respectively from Bergey's manual of Systematic Bacteriology [19].

The chitinase activity for CPB1 and CPB2 were estimated through chitinase activity assay. In that, the liberated N-acetyl glucosamine (Glycoprotein) was calculated through modified Protein estimation with reference to amine group, indirectly which gives the chitinase activity. CPB1 strain showed better activity (20µM/ml) after incubation of 6 hours (graph1).



Graph 1: Chitinase activity of two bacterial strains (CPB1 and CPB2)

The maximum activity of chitinase for CPB1 was observed at pH 8.0 and CPB2 showed moderate activity at pH 7.0 to 9.0 (Graph 2). Moreover combination of these both enzymes may increase the activity even in pH 7, 8 and 9.



Graph 2: Chitinase activity at different pH

The purification of extracellular chitinase from *Vibrio alginolyticus* and *Pseudomonas* sp. were characterized by SDS PAGE. Through gel documentation the molecular weights of two chitinase CPB1 and CPB2 were identified as 60KDa and 86KDa respectively (Figure 1).

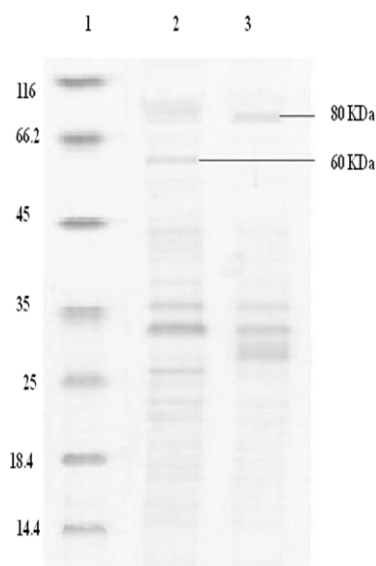


Figure 1: SDS-PAGE of purified chitinase: Lane1, marker; Lane2, CPB1; Lane3, CPB2.

DISCUSSION

Chitinase producing bacteria like, *Serratia marcescens*, *Bacillus circulans* [20] *Achromobacter*, *Pseudomonas*, *Flavobacterium* sp., *Alcaligenes denitrificans*, *Chromobacterium* and *Nocardia* genera have been reported [21]. Chitin in shrimp-shell waste was hydrolyzed by hydrochloric acid and given the yield of 80% of its weight of glucosamine [22]. It may affect the nature of aminoacid due to acid hydrolysis. However *Vibrio alginolyticus* have the ability to produce 259.2 U of enzyme (crude extracts) after incubation of 96 hours [23] and our results were inline with Donderski and Trzebiatowska (1999) [24]. This better enzyme activity may be due to the addition of $MnSO_4$ in the minimal media and reported that ions such as Mg^{2+} and Mn^{2+} could stimulate the chitinase and chitosanase activity [25].

The pH is the predominant condition for the production of enzymes. So the different in pH conditions were followed. Moreover the temperature under 35-40° C was suitable for enzyme production [10]. Other marine bacterial chitinases showed broader pH optima [10] and *Pseudomonas aeruginosa* K187 was highly active at acidic and alkaline pH [26]. Some authors reported that the molecular weights of chitinases from marine bacteria were mostly around 60KDa [10, 18]. However the molecular weight of chitinase enzyme produced by *Vibrio alginolyticus* was inlined with their results. From the crude extracts the results were found to be single molecule of chitinase but some authors reported that the chitinase having 2 or 3 molecules (Chi A, B and C as probes) [27].

Chitinase producing bacteria are predominantly present in shrimp ponds due to availability of chitin material in crustaceans and beneficial to other primary productive animals (microbes) which utilize N-acetyl glucosamine released from chitin degradation. The great variety of chitin structures which occurs in nature appears to necessitate that bacteria have a large number of different chitinase and other chitin-degrading enzymes. *Vibrio alginolyticus* and *Pseudomonas* sp produces different chitinases in response to the form of chitin they encounter, implying that they can distinguish among different types of chitin. Moreover chitinases also applicable as antifungal agents [28] and it may be produced through fermentation by using these strains with chitin as substrate.

Asia produces substantial amounts of marine waste, little of which is utilized efficiently. Effective utilization of such waste is not only good for the environment, but also increases the economic values of this marine and agricultural waste. Therefore recycling of waste into valuable products may decrease the livestock's feed economic rate like crude protein from feather keratin [29]. Therefore the

commercially available shrimp waste meal has little importance due to indigestion problem. By fermentation with these bacterial strains or its enzymes will cleave chitin to produce protein molecule and improve the digestion of livestock's. Moreover the extraction of carotenoids from shrimp waste through fermentation also reported [30].

CONCLUSION

Isolation and characterization of chitinase producing bacteria have the prime importance to degrade chitin and recycled as various by-products. Marine environment has a lot of source which fetch all the requirement to maintain the ecosystem. These chitinase in this environment can completely degrade the chitine waste to carbon and water. However we utilize the predominant bacterial strain and convert the waste into valuable protein products which will be used for fish or live stock's feed.

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